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Polyamines as a common source of hydrogen peroxide in host- and nonhost hypersensitive response during pathogen infection

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Abstract The hypersensitive response (HR) is a powerful resistance system that plants have developed against pathogen attack. There are two major pathways for HR induction; one is through recognition of the pathogen by a specific host protein, and is known as the host HR. The other is through common biochemical changes upon infection—the nonhost HR. We previously demonstrated that hydrogen peroxide derived from polyamine degradation by polyamine oxidase triggers the typical host HR in tobacco plants upon infection with tobacco mosaic virus. However, it remains to be determined whether or not polyamines are involved in the nonhost HR in tobacco, and in the host HR in other plant species. When tobacco plants were infected with *Pseudomonas cichorii*, a representative nonhost pathogen, transcripts for six genes encoding enzymes for polyamine metabolism were simultaneously induced, and polyamines were accumulated in apoplasts. Hydrogen peroxide was concomitantly produced and hypersensitive cell death occurred at infected sites. Silencing of polyamine oxidase by the virus-induced gene silencing method resulted in suppression of hydrogen peroxide production and in disappearance of visible hypersensitive cell death with an increase in bacterial growth. Our results indicated that polyamines served as the source of hydrogen peroxide during the nonhost HR in tobacco plants. Further analysis revealed that polyamines were accumulated in apoplasts of *Arabidopsis thaliana* infected with *Pseudomonas syringae*, and of rice infected with *Magnaporthe grisea*, both causing the typical host HR. As in tobacco, it is conceivable that the same mechanism operates for nonhost HR in these plants. Our present observations thus suggested that polyamines are commonly utilized as the source of hydrogen peroxide during host- and nonhost HRs in higher plants.

Keywords Hydrogen peroxide · Host HR · Nonhost HR · Polyamines · Polyamine oxidase · *Nicotiana benthamiana*

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Abbreviations

ADC	Arginine decarboxylase
DAB	3,3′-diaminobenzidine
HR	Hypersensitive response
Nb	*Nicotiana benthamiana*
Nt	*Nicotiana tabacum*
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
Put	Putrescine
SAMDC	*S*-Adenosylmethionine decarboxylase
Spd	Spermidine
SPDS	Spermidine synthase
Spm	Spermine

SPMS	Spermine synthase
TMV	Tobacco mosaic virus
TRV	Tobacco rattle virus
VIGS	Virus-induced gene silencing

Introduction

Plants have evolved specific defense systems to protect themselves against attack from a wide range of pathogens (Staskawicz et al. 1995; Heath 2000). Upon pathogen challenge, a necrotic lesion is formed at the site of pathogen entry, thereby preventing further spread of disease. This event is referred to as the hypersensitive response (HR).

The HR is initiated by recognition of pathogen attack, followed by an oxidative burst, induction of defense-related gene expression and hypersensitive cell death. There are two types of HR; one is cultivar-specific HR, in which a plant directly or indirectly recognizes the particular protein (avirulent protein) derived from the pathogen through the corresponding resistance (*R*) gene product (Hammond-Kosack and Jones 1997). This type of HR, often called host HR, is limited to a particular pathogen, and is referred to as a "gene-for-gene" mechanism (Keen 1990). Another type of HR is non-specific to particular pathogens. In this type of resistance, most plant species exhibit resistance to pathogens by responding to biochemical changes upon infection (Heath 2000). This type of HR, known as nonhost HR, is commonly observed in many plants in response to a variety of phytopathogens. As nonhost HR is effective against a broad spectrum of pathogens and is the predominant form of pathogen resistance among plants, its application might be useful to generate resistant crops. Its molecular mechanisms, however, are not yet completely understood, although similarities between host- and nonhost HRs have been documented. For example, an oxidative burst is induced during both HRs (Huckelhoven et al. 2001). Several defense signaling components functioning in host HR were also found in nonhost HR, including phytohormones (salicylic acid and ethylene) (Mellersh and Heath 2003; Knoester et al. 1998), MAP kinases (WIPK and SIPK) (Sharma et al. 2003), and ubiquitin ligase-associated protein SGT1 (Peart et al. 2002). It is thus conceivable that host- and nonhost HRs share at least some mechanisms.

Polyamines are small, positively charged aliphatic amines. They are involved in a multitude of cellular functions in plants, including growth, development and stress responses (Walden et al. 1997). Although the exact molecular basis of their biological function is still to be determined, their levels are known to fluctuate during plant–microbe interactions (Walters 2000). For example, during the incompatible interaction between barley and powdery mildew, polyamine levels greatly increased, accompanied by increased activity of the catabolic enzymes diamine oxidase and polyamine oxidase (Cowley and Walters 2002). Previously, we demonstrated that polyamines serve as the major source for hydrogen peroxide during host HR upon infection by tobacco mosaic virus (TMV) in tobacco plants carrying the resistant (*N*) gene (Yoda et al. 2003). We also showed that synthesized polyamines are efficiently degraded by polyamine oxidase to release hydrogen peroxide, which largely contributes to the second phase of the oxidative burst to induce hypersensitive cell death (Yoda et al. 2003, 2006). Thus, it was clear that polyamines, and their degradation product, hydrogen peroxide, were one of the key elements to induce host HR in tobacco plants.

Questions then arose whether or not a similar mechanism functions in nonhost HR in tobacco, and whether or not polyamines are commonly used during resistance in other plant species. In order to address these questions, we analyzed the interaction between tobacco plants (*Nicotiana benthamiana*) and its nonhost pathogen *Pseudomonas cichorii* (Sharma et al. 2003). We also examined the contribution of polyamines to host HR in *Arabidopsis* and rice. We show here that polyamine-derived hydrogen peroxide is critical not only for host HR but also for nonhost HR in tobacco plants, and that polyamines are commonly employed in other plant species in defense against pathogens.

Materials and methods

Plant materials and treatments

Tobacco (*Nicotiana benthamiana*) and rice (*Oryza sativa* cv Kinmaze) plants were grown in a growth cabinet at 23°C and 30°C, respectively, under a 14/10 h light/dark cycle. *Arabidopsis thaliana* (Colombia) was grown in a growth cabinet at 22°C under a 9/15 h light/dark cycle. *Pseudomonas cichorii* and *P. syringae* DC3000 (*avrRpm1*) were cultured in King's B medium containing 20 g/l Bacto peptone, 20 g/l Bacto tryptone, 20 g/l glycerol, 0.5 g/l K_2HPO_4 and 0.5 g/l KH_2PO_4, and diluted with 10 mM $MgCl_2$ ($OD_{600} = 0.01$ and 0.05, respectively) before inoculation. Tobacco leaves were infected with *P. cichorii* and *Arabidopsis* leaves were infected with *P. syringae* DC3000 (*avrRpm1*). Polyamines were infiltrated into tobacco and *Arabidopsis* leaves at a final concentration of 1 mM or 10 mM using a syringe without a needle, and leaves incubated at 23°C for appropriate time intervals under continuous light. Rice leaves were inoculated with *Magnaporthe grisea* (race 031) (1×10^5 conidia/ml) as described (Iwai et al. 2006) and incubated at 30°C under a 14/10 h light/dark cycle. Polyamines were absorbed at the

cutting site of rice leaves and incubated at 30°C for appropriate time intervals under continuous light.

Polyamine quantification

Apoplastic fluids were extracted from leaf disks prepared from tobacco or *Arabidopsis* (15 mm in diameter), or from rice leaf segments (2–3 cm in length). Each leaf disk or leaf segment was weighed and submerged in water *in vacuo*. Leaf extract was collected by a brief centrifugation by placing samples in a 10-ml syringe, which was set in a 50-ml Falcon tube. Amines in extracted fluids were quantified either by HPLC as previously described (Yoda et al. 2003) or by capillary electrophoresis mass spectrometry (Soga and Heiger 2000).

RNA gel-blot analysis

Total RNA was isolated from leaf samples by the guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987), and gel-blot hybridization was performed as previously described (Yoda et al. 2002). Probes for each gene were synthesized with a pair of specific primers, which were designed based on the cDNA from *N. tabacum*: forward (5′-GGCTCATTACCAAGGTGTTTATCC-3′) and reverse (5′-CCAAGCCCACCAATATCAATG-3′) for *NbADC*; forward (5′-TCAGCTTGGATAAGAATAAGCGAGG-3′) and reverse (5′-ATGGCCGGCCAAACAATCATCG-3′) for *NbODC*; forward (5′-GGCCTTGCCTGTCTCTGCCATTGG-3′) and reverse (5′-TTGGTAGCAACATCAGCATGCAAAGC-3′) for *NbSAMDC*; forward (5′-TGAGATTAGCCCCCTTTGGCCTGGGG-3′) and reverse (5′-GCAAATGATGGCAAACAGAATGATGC-3′) for *NbSPDS*; forward (5′-GGAAAAGTTCTAGTGCTAGATGG-3′) and reverse (5′-GGATCTGATGAATCAACTATGATAGC-3′) for *NbSPMS*; and forward (5′-AAGAAAGCGGTGGACTCGGC-3′) and reverse (5′-TCGTGGGCGTAGATGAAGAACTCC-3′) for *NbPAO*.

Virus-induced gene silencing

Using cDNA derived from *P. cichorii*-infected *N. benthamiana* leaves as a template, a partial fragment of *NbPAO* was amplified by PCR with a pair of synthesized primers; forward containing *Bam*HI site (5′-GGATCCAAGAAAGCGGTAGACTCGGC-3′) and reverse containing *Kpn*I site (5′-GGTACCTCGTGGGCGTAGATGAAGAAC-3′). The resulting fragment was introduced into corresponding sites of a pTV00 vector derived from Tobacco rattle virus (TRV), which was transformed into *A. tumefaciens* GV3101 strain. The resultant GV3101 strain and *A. tumefaciens* C58C1 strain containing pBINTRA6 (Ratcliff et al. 2001) were grown to saturation in LB medium. Cells were collected by centrifugation and resuspended in a solution containing 10 mM $MgCl_2$, 10 mM MES and 100 μM acetosyringone ($OD_{600} = 0.5$), and kept at room temperature for 2 h. Each culture containing pBINTRA6 or pTV00-derived constructs (or pTV00 as a control vector) was mixed in a 1:1 ratio, and infiltrated into a lower leaf of 3-week old tobacco plants using a syringe without a needle. Infiltrated tobacco plants were incubated at 25°C under a 15/9 h light/dark cycle for 3 weeks and used for further experiments.

Detection of hydrogen peroxide

Leaf samples infected with pathogen or infiltrated with polyamines were incubated for indicated time intervals and then submerged in 3,3′-diaminobenzidine (DAB) solution (1 mg/ml, pH 3.8) for 8 h. DAB deposits indicative of DAB oxidation products were visualized after brief boiling in ethanol as previously described (Yoda et al. 2003).

Bacteria growth assay

A leaf disk (15 mm in diameter) from infected leaves with *P. cichorii* was ground in 10 mM $MgSO_4$ solution. The resultant crude extract was diluted to the appropriate concentration, and spread onto King's B medium. Bacterial numbers in leaves were calculated from colony units formed on the medium.

DNA fragmentation assay

DNA was extracted from leaves infected with *P. cichorii* by the cetyltrimethyl ammonium bromide method (Murray and Thompson 1980), fractionated on a 2% agarose gel and visualized by staining with ethidium bromide as previously described (Yoda et al. 2006).

Estimation of cell death

Leaf samples treated with amines (infiltrated or absorbed) were submerged in a solution containing 2.5 mg/ml trypan blue, 25% lactic acid, 23% acid phenol, and 25% glycerol at 70°C for 15 min in vacuo. This step was repeated twice. After boiling for 2 min, samples were incubated at room temperature for 1 h. Samples were recolorized with chloral hydrate (2.5 g/ml) several times and fixed with 70% glycerol to visualize stained regions.

Results

Polyamine accumulation during nonhost HR

In our previous studies with TMV and tobacco plants (*N. tabacum*) carrying the resistant (*N*) gene, by which the

host HR is induced, we demonstrated that polyamines were accumulated in apoplasts of infected leaves, and degraded by polyamine oxidase, releasing hydrogen peroxide that causes hypersensitive cell death (Yoda et al. 2003). In the present study, we examined whether or not polyamines similarly function during the nonhost HR. *P. cichorii* was previously reported to induce a nonhost HR on *N. benthamiana* (Sharma et al. 2003). When bacteria were inoculated onto healthy leaves of *N. benthamiana*, an HR lesion began to appear approximately 12 h later, and hypersensitive cell death was evident at ~24 h (Fig. 1a). To determine whether or not polyamines were involved in this response, apoplastic fluids were collected and subjected to the HPLC analysis. Putrescine was found to be present in apoplasts of intact leaves at a low level, and increased up to 10-fold 24 h after infection (Fig. 1b). Spermidine was scarcely detectable before infection, but began to accumulate after infection up to 24 h (Fig. 1b). The control plants infiltrated only with $MgCl_2$ solution (mock treatment) also exhibited a slight increase of both amines, this being probably due to infiltration stress (Fig. 1b). The amount of polyamines in intracellular space was then examined. The basal level of putrescine was high, showing approximately 80 nmole per gram fresh weight, and increased up to 3-fold in infected leaves 24 h later (Fig. 1c). In control samples (mock-treated leaves), such an increase was not observed (Fig. 1c). The basal level of spermidine was also high, showing 100 nmole per gram fresh weight, and this level was maintained for 24 h in treated and untreated leaves (Fig. 1c). Spermine was not detectable at any experimental time points in treated and untreated leaves in both apoplasts and intracellular spaces (data not shown). To take account of amines from *P. cichorii*, the amount of each amine was examined in excess *P. cichorii* (1×10^8 cfu). The amount of spermidine derived from *P. cichorii* was 0.92 ± 0.05 nmol, while those of putrescine and spermine were undetectable. This result indicated that amine compounds derived from *P. cichorii* were negligible to estimate the total amine contents in inoculated leaves.

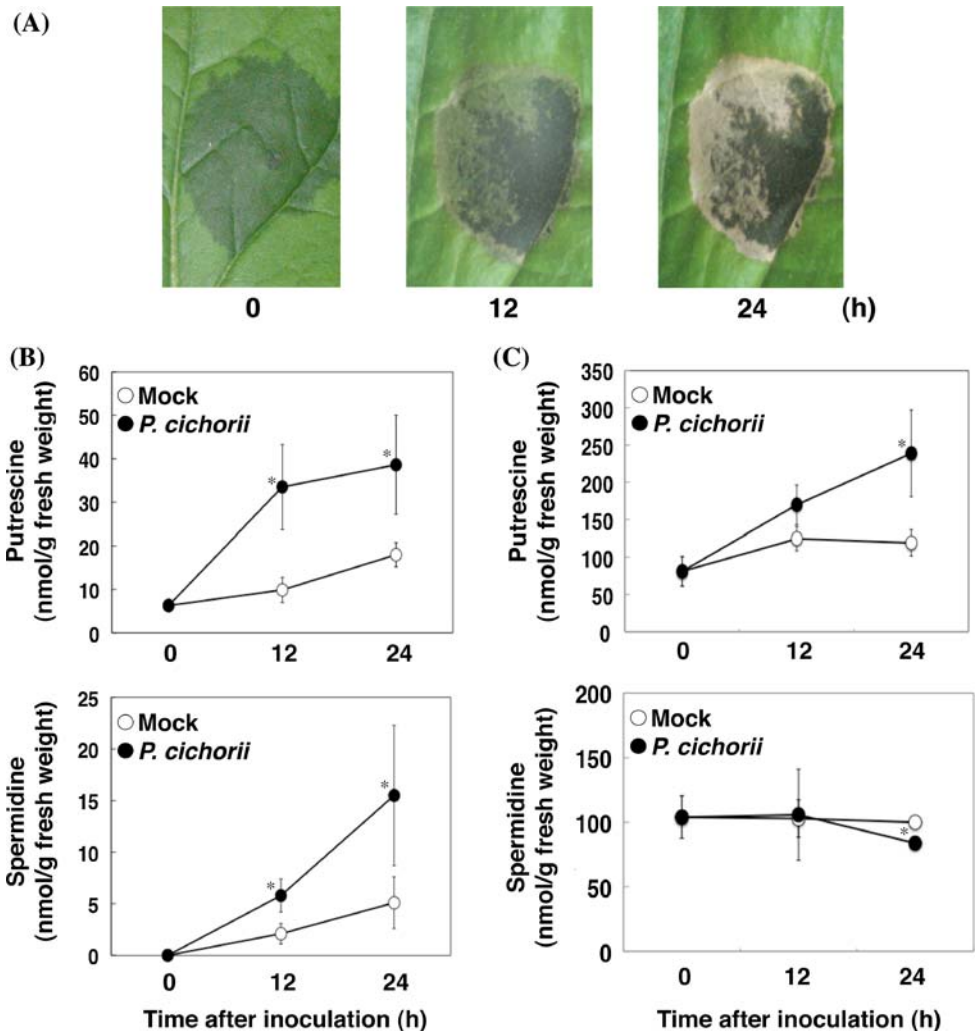


Fig. 1 Nonhost HR and polyamine accumulation. **a** Formation of necrotic lesion. A healthy leaf of wild-type tobacco (*N. benthamiana*) was infiltrated with *P. cichorii*, and observed for visible necrotic lesion formation after 12 h and 24 h. **b**, **c** Accumulation of putrescine and spermidine in apoplast (**b**) and in intracellular space (**c**). Apoplastic fluid was extracted from leaves infected with *P. cichorii* (closed circle) or mock-treated (open circle) at indicated time points. Intracellular amines were extracted from remaining leaf-disks after extraction of apoplastic fluid. Values are means from four independent assays with standard deviations with the significance of difference of the 95% confidence intervals ($P < 0.05$)

Induction of transcripts for polyamine metabolic enzymes

Polyamines are synthesized and catabolized through multiple steps, each step being catalyzed by a specific enzyme. To examine expression profile, cDNAs encoding these enzymes were newly isolated, and designated as *NbADC*, *NbODC*, *NbSAMDC*, *NbSPDS*, *NbSPMS* and *NbPAO*. When healthy leaves were challenged by the nonhost pathogen, *P. cichorii*, all transcripts for these genes were simultaneously induced (Fig. 2). Transcripts for *NbADC*, *NbODC*, *NbSAMDC*, *NbSPDS* and *NbPAO* began to accumulate 6 h after infection, reaching a plateau 12 h later with a gradual decline thereafter (Fig. 2). Transcripts for *NbSPMS* were low and began to accumulate 12 h after infection (Fig. 2). These results suggest that increase of amines during the nonhost HR is due to an increase in expression of the relevant genes.

Hydrogen peroxide production by polyamine oxidase

Polyamine oxidase is essential for generating hydrogen peroxide to induce programmed cell death during the host HR (Yoda et al. 2003). To investigate the possibility that polyamine oxidase is also required in the nonhost HR, the virus-induced gene silencing (VIGS) method was employed to transiently silence the gene encoding polyamine oxidase from *N. benthamiana* (*NbPAO*). A partial cDNA fragment for *NbPAO* was isolated with a pair of primers, which were designed based on the cDNA for a polyamine oxidase from *N. tabacum* (*NtPAO*). The deduced polypeptide with 188 amino acids of NbPAO was similar to that of NtPAO (Fig. 3a), indicating the isolated cDNA was an ortholog in *N. benthamiana*. Total suppression of *NbPAO* expression was confirmed by RT-PCR (Fig. 3b). Using these materials, we first directly infiltrated polyamines into leaves, and observed lesion formation. In wild-type plants, spermidine and spermine at a concentration of 1 mM induced HR-like cell death 72 h and 24 h after infiltration, respectively, whereas putrescine did not (Fig. 4a). The effect appeared to be dose-dependent, as HR-like cell death was observed faster and clearer upon infiltration with 10 mM polyamines in comparison with 1 mM (Fig. 4a). Hence we performed further experiments with a fixed concentration of amines at 10 mM. In *NbPAO*-silenced tobacco plants, hydrogen peroxide was not produced and cell death was not induced either (Fig. 4b). Subsequently, we examined effects of *P. cichorii* infection. In wild-type plants, hydrogen peroxide was produced and hypersensitive cell death was induced (Fig. 4c). In *NbPAO*-silenced tobacco plants, neither hydrogen peroxide nor cell death was observed (Fig. 4c). The number of bacteria grown during infection was then examined. When 10^7 cfu/ml of *P. cichorii* was used as the inoculum, the number equally increased up to 2×10^8 cfu/cm^2 during the first 2 days in both control and *NbPAO*-silenced plants. However, the growth thereafter was markedly suppressed in the control plants due to the nonhost HR, whereas growth in *NbPAO*-silenced plants increased up to 6×10^8 cfu/cm^2 5 days after infection (Fig. 4d). Further analysis indicated that, upon *P. cichorii* infection, genomic DNA of wild-type plants was markedly degraded, showing fragmentation patterns, while that of *NbPAO*-silenced plants apparently remained intact (Fig. 5). These results

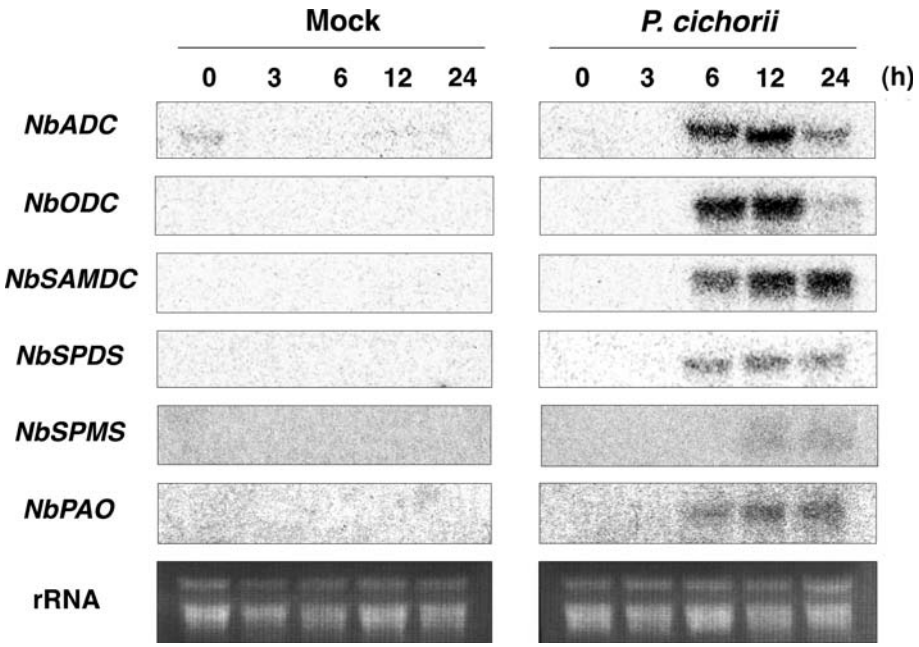


Fig. 2 Accumulation profile of transcripts for enzymes involved in polyamine metabolism during the nonhost HR. Healthy leaves of wild-type tobacco were treated with *P. cichorii* or with water (mock-treated) for indicated time period. Total RNA was extracted from leaves, and subjected to RNA gel-blot analysis with the indicated probe labeled with ^{32}P. rRNA was used as loading control. Nucleotide sequence data reported here are available in the DDBJ/EMBL/GenBank databases under the accession numbers: *NbADC*; AB304777, *NbODC*; AB304778, *NbSAMDC*; AB304782, *NbSPDS*; AB304779, *NbSPMS*; AB304780, and *NbPAO*; AB304781

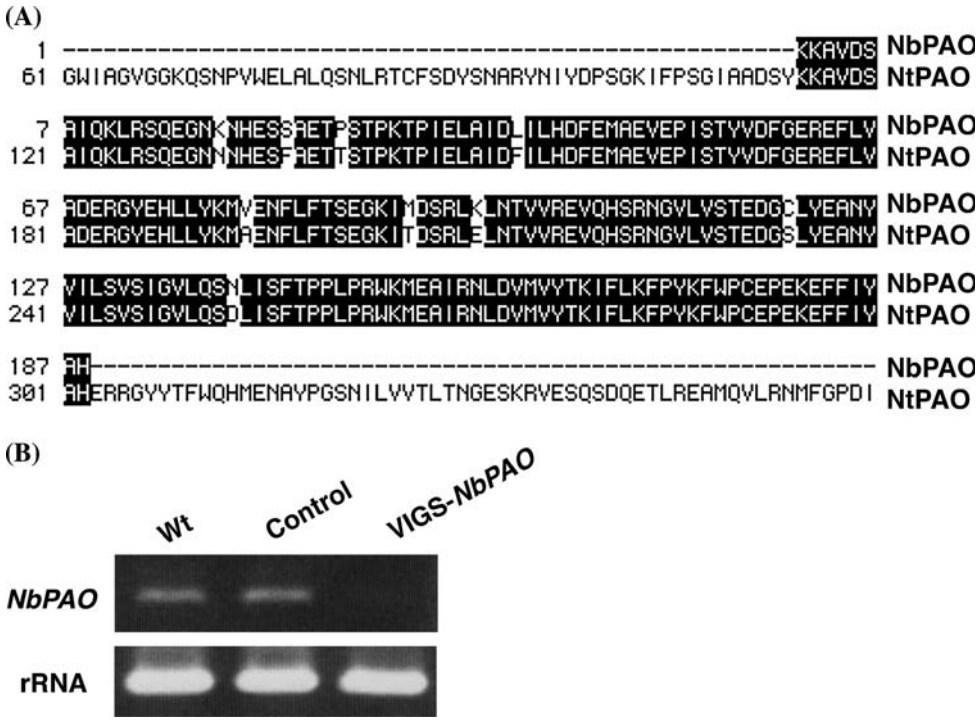


Fig. 3 Construction of *NbPAO* silencing tobacco. **a** Partial amino acid sequence alignment of NbPAO with NtPAO (accession no. AB200262). Numbers on the left side indicate amino acid position. **b** Confirmation of VIGS silencing. Three-week old tobacco plants (*N. benthamiana*) were infected with *A. tumefaciens* (GV3101 strain) containing the pTV00-*NbPAO* construct or empty vector (control) and cultivated under standard conditions. Leaves were detached from control (control) or *NbPAO*-silenced (VIGS-*NbPAO*) plants 3 weeks later and infected with *P. cichorii*. Total RNA was extracted from each infected leaf 24 h after infection and subjected to RT-PCR. rRNA was used as the internal control

strongly suggested that hypersensitive cell death took place in the wild-type but not in the transgenic plants.

Polyamines in other plant species

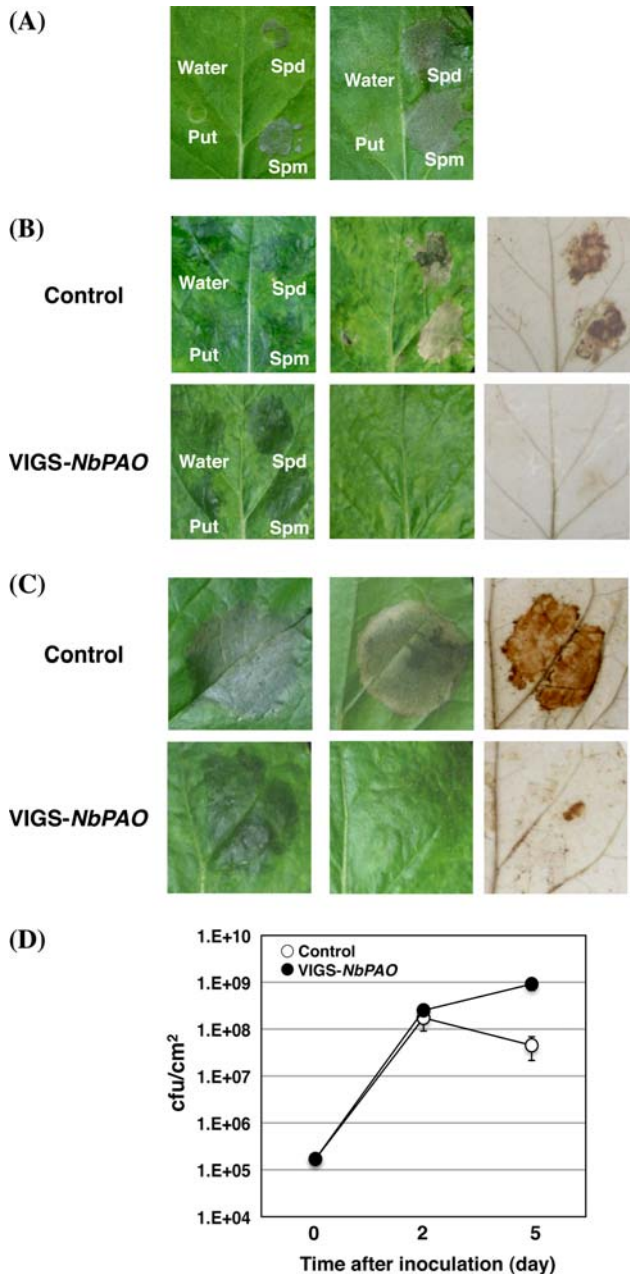
The above-mentioned experiments indicated that polyamines are a direct substrate for hydrogen peroxide production in both the host- and nonhost HRs in tobacco plants. We then aimed to determine whether polyamines similarly function in the HR of other plant species. Healthy leaves from *Arabidopsis* (dicot) and rice (monocot) plants were inoculated with respective host pathogens, *P. syringae* DC3000 (*avrRpm1*) and *M. grisea* (race 031), and examined for polyamine production. Results showed that putrescine and spermidine accumulated in apoplasts after inoculation in both plant species (Fig. 6a, b). In *Arabidopsis*, the basal levels of putrescine and spermidine were low and reached 12 nmol and 11 nmol per gram fresh weight 24 h after inoculation, respectively (Fig. 6a). In rice, putrescine was hardly detectable before inoculation, but increased to more than 10 nmol per gram fresh weight 21 days after inoculation (Fig. 6b). The basal level of spermidine in rice was undetectable, but began to increase upon infection, reaching 1.7 nmol per gram fresh weight 7 days later (Fig. 6b). This level was maintained up to 21 days, although its total amount was lower than that of putrescine. The absolute level of spermidine in rice was 1/7 that of the level in *Arabidopsis* (Fig. 6a, b). Polyamine level in rice was undetectable under mock-treated leaves (data not shown).

Induction of hydrogen peroxide and cell death by polyamines

Effects of exogenously applied polyamines were then examined in a similar manner as performed with tobacco plants (Fig. 4b). Healthy leaves from *Arabidopsis* and rice plants were infiltrated with putrescine, spermidine or spermine, and hydrogen peroxide production and cell death were analyzed. Results showed that spermidine and spermine efficiently induced production of hydrogen peroxide and concomitant cell death in both plants, whereas putrescine did not (Fig. 6c, d).

Discussion

Plants defend themselves against the majority of pathogens through the combination of host HR and nonhost HR systems. Since the host HR is usually race-specific, it is restricted to particular pathogen species. In contrast, the nonhost HR is observed across all plant species, and is effective against a broad spectrum of pathogens. It is more common and more durable than the host HR (Mysore and Ryu 2004). In this article, we documented that polyamine-derived hydrogen peroxide catalyzed by polyamine oxidase is sufficient to induce the hypersensitive cell death during the nonhost HR in tobacco plants. This observation is consistent with the case of the host HR against TMV (Yoda et al. 2003), suggesting that both host- and nonhost HRs



◀ **Fig. 4** Effects of *NbPAO* silencing on the nonhost HR. **a** Lesion development by polyamine infiltration. Healthy leaves of wild-type (WT) tobacco were infiltrated with water or indicated amines at 1 mM (left panel) or 10 mM concentration (right panel) using a syringe without a needle. Necrotic lesion due to cell death was observed 72 h (1 mM) and 24 h (10 mM) after infiltration, respectively. *Put* putrescine, *Spd* spermidine, *Spm* spermine. **b** Polyamine-induced cell death and hydrogen peroxide production. Healthy leaves of wild-type tobacco treated with empty vector (control, upper panel) or *NbPAO*-silenced plants (VIGS-*NbPAO*, lower panel) were infiltrated with water or indicated amines (left panel). A necrotic lesion was observed 24 h later (middle panel), and hydrogen peroxide production was observed 12 h later (right panel). **c** Pathogen-induced cell death and hydrogen peroxide production. Healthy leaves of wild-type tobacco treated with empty vector (control, upper panel) or *NbPAO*-silenced plants (VIGS-*NbPAO*, lower panel) were infected with *P. cichorii* (left panel), and nonhost hypersensitive cell death was observed 24 h later (middle panel). Hydrogen peroxide production was observed 12 h after infection (right panel). **d** Bacterial population in inoculated region. Healthy leaves of control (Control; open circle) or *NbPAO*-silenced plants (VIGS-*NbPAO*; closed circle) were infected with *P. cichorii*, and the number of bacteria determined at indicated time points. Values are means from three independent assays with standard deviations

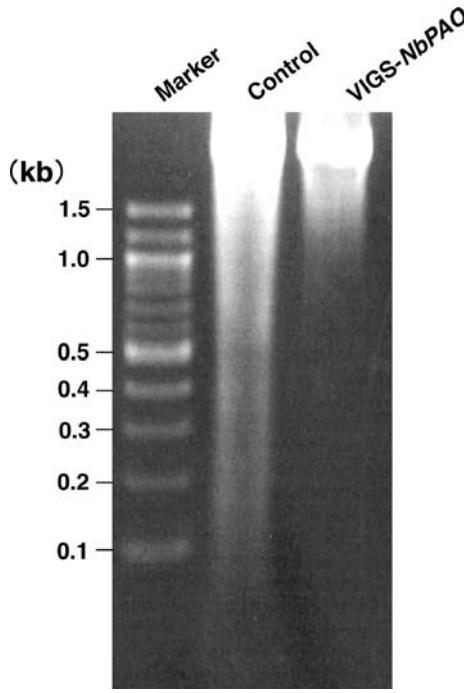


Fig. 5 DNA fragmentation. Healthy leaves of wild-type tobacco treated with the empty vector (control) or *NbPAO*-silenced plants (VIGS-*NbPAO*) were infected with *P. cichorii*, and genomic DNA was extracted 24 h later. After fractionation by 2% agarose gel electrophoresis, DNA was visualized by staining with ethidium bromide

share a common system utilizing polyamines as the source of hydrogen peroxide.

Experimentally we showed that silencing of *NbPAO* results in the suppression of hydrogen peroxide production and eventually of the HR onset. We also found that the in vivo levels of putrescine and spermidine are temporally and spacially controlled during bacterial infections. Spermidine and spermine, but not putrescine, are considered to be substrates of NbPAO as in the case for NtPAO (Yoda et al. 2003), both showing a close similarity in sequence. Although the in vivo substrates of PAO from the various organisms may be diverse, including polyamine derivatives and conjugates, we speculated that in *N. benthamiana* spermidine and spermine possibly serve as a source of hydrogen peroxide during the HR through their oxidation. Recent findings have shown that in Arabidopsis spermidine and spermine are oxidized through a polyamine back-conversion pathway resulting in the production of putrescine

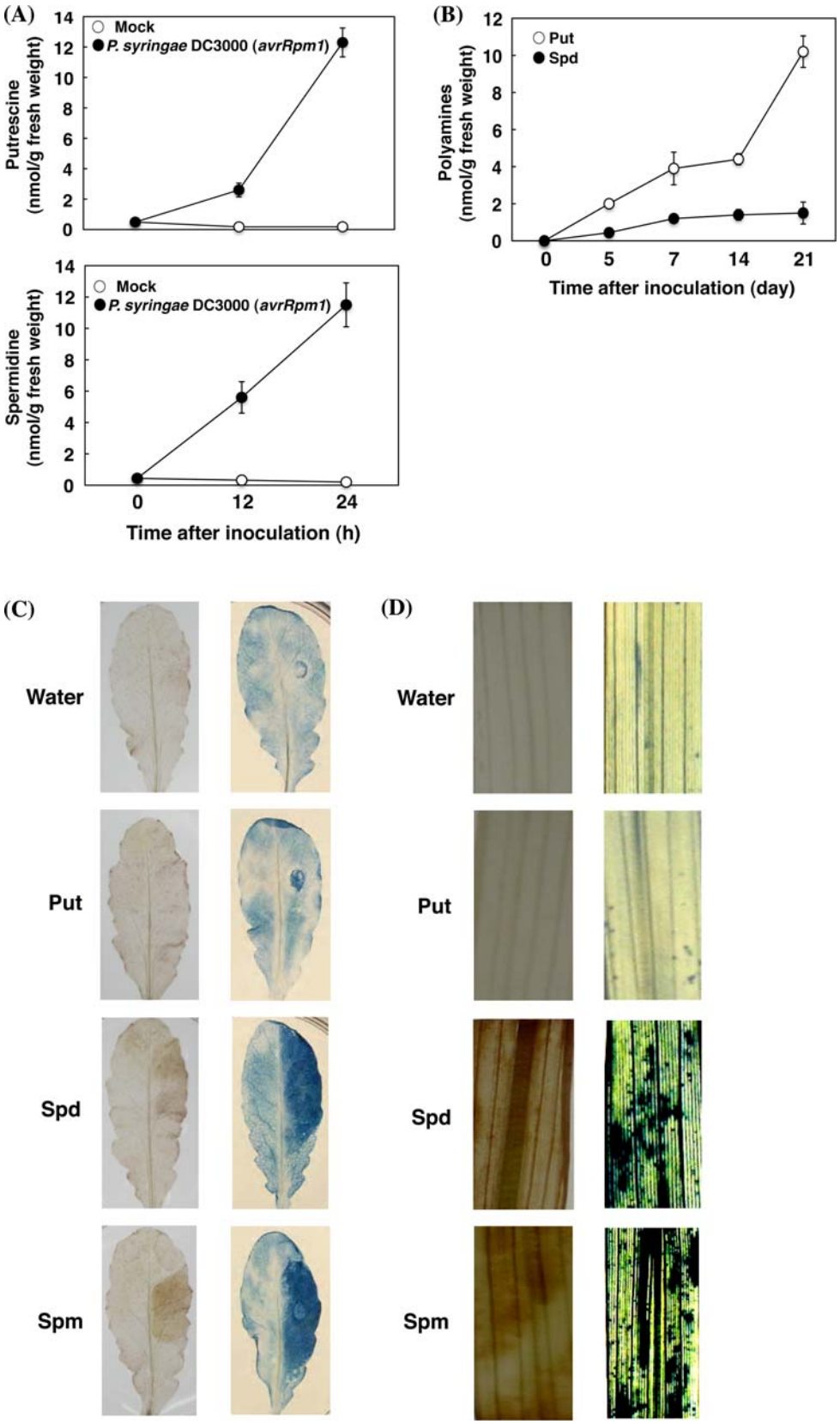


Fig. 6 Requirement of polyamines for HR induction in *Arabidopsis* and rice. **a** Accumulation profile of amines in *Arabidopsis*. Healthy leaves of *Arabidopsis* were inoculated with (closed circle) or without (open circle) the host pathogen, *P. syringae* DC3000 (*avrRpm1*), and amounts of putrescine (upper panel) and spermidine (lower panel) in apoplasts was determined. Values are from three independent assays with standard deviations. **b** Accumulation profile of amines in rice. Healthy leaves of rice plants were inoculated with the host pathogen *M. grisea* (race 031), and amounts of putrescine (open circle) and spermidine (closed circle) in apoplasts were determined at indicated time points. Values are from three independent assays with standard deviation. **c**, **d** Polyamine-induced cell death in *Arabidopsis* (**c**) and in rice (**d**). Indicated amine compounds were infiltrated into leaves, and hydrogen peroxide production (left panel) and cell death (right panel) were observed 12 h and 24 h later, respectively. *Put* putrescine, *Spd* spermidine, *Spm* spermine

and spermidine, respectively (Tavladoraki et al.; Moschou et al. 2008; Kamada-Nobusada et al. 2008). Thus, in the case of tobacco plants, putrescine and spermidine accumulated in apoplast are highly probable to be produced through this pathway. This idea accounts for the undetectable level of spermine in apoplasts, suggesting its complete back-conversion to spermidine by NbPAO. Although spermine was reported to be accumulated in extracellular space during HR in tobacco plants upon TMV infection (Yamakawa et al. 1998), the difference might be due to the different protocols for polyamine extraction from the extracellular space, being under non denaturing conditions in our present work. Since polyamine oxidase from barley was reported to be in vacuole and that from *Ararbidopsis* in peroxisome (Cervelli et al. 2004; Moschou et al. 2008; Kamada-Nobusada et al. 2008), it is conceivable that polyamine dependent production of hydrogen peroxide also takes place in the intracellular space. It remains to be determined, however, whether or not thus-produced hydrogen peroxide contributes to the HR. Putrescine is generally considered to be degraded by copper amine oxidases (CuAO) (Cona et al. 2006), but in the present case, accumulated putrescine appeared not to be degraded, since *NbPAO*-silenced plants did not produce detectable level of hydrogen peroxide when infiltrated with putrescine. This suggested the absence of CuAO activity in apoplasts during the early phase of nonhost HR, being consistent with a previous report showing the absence of extracellular enzymes catabolizing putrescine in tobacco protoplasts (Papadakis and Roubelakis-Angelakis 2005). Since putrescine is known to serve as an antioxidant (Groppa and Benavides 2008), and/or to prevent programmed cell death syndrome (Papadakis and Roubelakis-Angelakis 2005), one of the biological functions of accumulated putrescine could be to prevent excess hypersensitive cell death.

Expression of genes for polyamine metabolism is temporally controlled, transcripts beginning to accumulate 6 h after inoculation. This induction profile is similar to our previous observation on the host HR (Yoda et al. 2003). It is also consistent with our previous observation that polyamine-derived hydrogen peroxide is generated at the late phase of the oxidative burst in BY2 cells that were elicited by cryptogein (Yoda et al. 2006). Such a late and simultaneous induction of gene expression suggested that some upstream signaling molecule(s) are involved. Methyl jasmonate is one of the candidates, as it has been shown to induce expression of *ODC*, *ADC*, *SAMDC* and *SPDS*, whereas these are not induced by salicylic acid or ethylene (Imanishi et al. 1998; Walters et al. 2002; Yoda unpublished observation).

In the host HR, we proposed that, upon pathogen attack, genes for polyamine metabolism are transcriptionally activated. This results in biosynthesis and accumulation of polyamines in apoplasts, and the polyamine-derived hydrogen peroxide directly contributes to hypersensitive cell death (Yoda et al. 2003, 2006). In the nonhost HR, we also found that hydrogen peroxide generated from polyamines is the major signaling molecule for inducing hypersensitive cell death. These observations strongly suggest that the induction mechanism of the oxidative burst is common between the host- and nonhost HRs. Since other sources for oxidative burst, for instance NADPH oxidase, have also been reported for both host- and nonhost HRs (Bestwick et al. 1998), the oxidative burst could possibly be differentially regulated in temporal and spatial manners during hypersensitive cell death.

A notable feature of the present study is that polyamines accumulated in apoplasts of *Arabidopsis* and rice during the host HR, and that exogenously applied polyamines induced HR-like cell death in both plant species. These findings are consistent with our previous observation in tobacco plants (Yoda et al. 2003). It appears that polyamines are commonly utilized among plant species, although the accumulation profiles and amounts of polyamines varied in each plant species. The level of putrescine was comparable in both plants, although its accumulation rate in rice was much slower than in *Arabidopsis*. The level of spermidine in *Arabidopsis* increased after pathogen infection, showing a similar profile as in tobacco. In contrast, its level in rice was lower and increasing rate was slower than in *Arabidopsis* and tobacco plants. The low level of spermidine in rice suggests that rice may be more sensitive to low levels of hydrogen peroxide in comparison with *Arabidopsis* and tobacco plants. It is also conceivable that the kinetics of polyamine metabolism varies depending on the pathogen species; virus, bacteria and fungus, of which infection mode differs from each other. Overall, we propose that polyamines are the common source for hydrogen peroxide during the host HR among plant species. It is also tempting to speculate that, judging from the case in tobacco plants, polyamines may also commonly serve as the source for hydrogen peroxide in the nonhost HR in several plant species.

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